



Europäisches Patentamt  
European Patent Office  
Office européen des brevets



⑪ Publication number: 0 498 537 A2

⑫

## EUROPEAN PATENT APPLICATION

⑬ Application number: 92300366.9

⑭ Int. Cl.<sup>5</sup>: C12N 15/32, A01N 63/02,  
C12N 1/20, C12P 1/04,  
C12N 1/21, // (C12P1/04,  
C12R1:07), (C12N1/21,  
C12R1:19, 1:39)

⑯ Date of filing: 16.01.92

The microorganism(s) has (have) been deposited with NRRL under number(s) B-18746 and B-18751.

⑮ Priority: 16.01.91 US 642112

⑯ Inventor: Foncarrada, Luis  
2735 Tereita  
San Diego, California 92104 (US)  
Inventor: Sick, August J.  
318A San Helena Drive  
Oceanside, California 92056 (US)  
Inventor: Payne, Jewel M.  
7984 Hemphill Drive  
San Diego, California 92126 (US)

⑰ Date of publication of application:  
12.08.92 Bulletin 92/33

⑯ Representative: Perry, Robert Edward  
GILL JENNINGS & EVERY 53-64 Chancery  
Lane  
London WC2A 1HN (GB)

⑲ Designated Contracting States:  
AT BE CH DE DK ES FR GB GR IT LI LU MC NL  
PT SE

⑳ Applicant: Mycogen Corporation  
5451 Oberlin Drive  
San Diego, CA 92121 (US)

㉑ Novel coleopteran-active bacillus thuringiensis isolate and a novel gene encoding a coleopteran-active toxin.

㉒ The subject invention concerns a novel microbe and gene encoding a novel toxin protein with activity against insect pests of the order Coleoptera. Pests in the order Coleoptera do heavy damage to crops, e.g., corn. The novel *Bacillus thuringiensis* microbe of the invention is referred to as B1. PS50C. The spores or crystals of this microbe, or mutants thereof, are useful to control coleopteran pests in various environments. The novel gene of the invention can be used to transform various hosts wherein the novel toxic protein can be expressed.

EP 0 498 537 A2

Background of the Invention

*Bacillus thuringiensis* (B.t.) produces an insect toxin designated as δ-endotoxin. It is synthesized by the B.t. sporulating cell. The toxin, upon being ingested in its crystalline form by susceptible insect larvae, is transformed into biologically active moieties by the insect gut juice proteases. The primary target is insect cells of the gut epithelium, which are rapidly destroyed.

The reported activity spectrum of B.t. covers insect species within the order Lepidoptera, many of which are major pests in agriculture and forestry. The activity spectrum also includes the insect order Diptera, which includes mosquitoes and black flies. See Couch, T.L. (1980) "Mosquito Pathogenicity of *Bacillus thuringiensis* var. *lengaeensis*," Developments in Industrial Microbiology 22:61-76; Beegle, C.C., (1978) "Use of Entomogenous Bacteria in Agroecosystems," Developments in Industrial Microbiology 20:97-104. Krieg, et al., Z. ang. Ent. (1983) 96:500-508, describe a B.t. isolate named *Bacillus thuringiensis* var. *tenebrionis*, which is reportedly active against two beetles in the order Coleoptera. These are the Colorado potato beetle, *Leptinotarsa decemlineata*, and *Agalathid atra*.

In European Patent Application 0 202 739 there is disclosed a novel B.t. isolate active against Coleoptera. It is known as *B. thuringiensis* var. *san diego* (B.t.sd.). U.S. Patent No. 4,966,765 discloses the coleopteran-active *Bacillus thuringiensis* isolate B.t. PS86B1. European Patent Application 0 337 604 also discloses a novel B.t. isolate active against Coleoptera. This isolate is B.t. PS43F.

Coleopteran-active strains, such as B.t.sd., B.t. PS86B1, and B.t. PS43F, can be used to control foliar-feeding beetles. The Colorado potato beetle (*Leptinotarsa decemlineata*), for example, is susceptible to the delta-endotoxin of B.t.sd. and larvae are killed upon ingesting a sufficient dose of spore/crystal preparation on treated foliage.

A number of crops are attacked by flea beetles. These beetles belong to the family Chrysomelidae, the decemlineatae. The adults can cause extensive damage by feeding on the foliage.

Brief Summary of the Invention

The subject invention concerns a novel *Bacillus thuringiensis* (B.t.) isolate and a cloned gene therefrom which encodes a novel coleopteran-active protein. The novel B.t. isolate, known herein as *Bacillus thuringiensis* PS50C (B.t. PS50C), has thus far been shown to be active against the Colorado potato beetle (*Leptinotarsa decemlineata*). The novel δ-endotoxin gene of the invention encodes an ≈130 kDa protein. The nucleotide sequence of the gene (open reading frame only) is shown in Sequence ID No. 1. The predicted peptide sequence of the toxin is shown in Sequence ID No. 2.

The subject invention also includes mutants of B.t. PS50C which have substantially the same pesticidal properties as B.t. PS50C. Procedures for making mutants are well known in the microbiological art. Ultraviolet light and nitrosoguanidine are used extensively toward this end.

Further, the invention also includes the treatment of substantially intact B.t. PS50C cells, and recombinant cells containing the gene of the invention, to prolong the pesticidal activity when the substantially intact cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or a combination of chemical or physical means, so long as the technique does not deleteriously affect the properties of the pesticide, nor diminish the cellular capability in protecting the pesticide. The treated cell acts as a protective coating for the pesticidal toxin. The toxin becomes available to act as such upon ingestion by a target insect.

Detailed Disclosure of the Invention

The novel *Bacillus thuringiensis* isolate of the subject invention has the following characteristics in its biologically pure form:

Characteristics of B.t. PS50C

- 50 Colony morphology—Large colony, dull surface, typical B.t.
- Vegetative cell morphology—typical B.t.
- Culture methods—typical for B.t.
- Flagellar serotyping—PS50C belongs to serotype 18, kumamotoensis.
- Crystal morphology—a sphere.
- RFLP analysis—Southern hybridization of total DNA distinguishes B.t. PS50C from B.t.sd. and other B.t. isolates.
- Alkali-soluble proteins—SDS polyacrylamide gel electrophoresis (SDS-PAGE) shows a 130 kDa doublet protein.

A comparison of the characteristics of B. thuringiensis PS50C (B.t. PS50C) to the characteristics of the known B.t. strains B. thuringiensis var. san diego (B.t.sd.), B. thuringiensis PS86B1 (NRRL B-18299), and B. thuringiensis var. kurstaki (HD-1) is shown in Table 1.

5

**Table 1.** Comparison of B.t. PS50C, B.t. PS86B1, B.t.sd., and B.t. HD-1

	<u>B.t.</u> PS50C	<u>B.t.sd.</u>	<u>B.t.</u> PS86B1	<u>B.t.</u> HD-1
Serovar	kumamotoensis	morrisoni	tolworthi	kurstaki
Type of inclusion	sphere	square wafer	flat, pointed ellipse, plus sm. inclusions	Bipyramidal
Size of alkali-soluble proteins by SDS-PAGE	130 kDa doublet	72,000 64,000	75,000 68,000 61,000	130,000 68,000
Host range	Coleoptera	Coleoptera	Coleoptera	Lepidoptera

25

The cultures disclosed in this application have been deposited in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, USA.

30

<u>Culture</u>	<u>Repository No.</u>	<u>Deposit date</u>
<u>Bacillus thuringiensis</u> PS50C	NRRL B-18746	January 9, 1991
<u>Escherichia coli</u> NM522 [pMYC1638]	NRRL B-18751	January 11, 1991

40

B.t. PS50C, NRRL B-18746, can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle, the bacteria can be harvested by first separating the B.t. spores and crystals from the fermentation broth by means well known in the art. The recovered B.t. spores and crystals can be formulated into a wettable powder, liquid concentrate, granules, or other formulations by the addition of surfactants, dispersants, inert carriers and other components to facilitate handling and application for particular target pests. These formulation and application procedures are all well known in the art.

45

Plasmid DNA (pMYC1638) containing the toxin gene from B.t. PS50C can be purified from E. coli NM-522(pMYC1638) by standard procedures well known in the art. The toxin gene can be excised from the plasmid DNA by restriction enzyme digestion, as indicated in the accompanying drawing.

50

Formulated products can be sprayed or applied onto foliage to control phytophagous beetles or caterpillars.

Another approach that can be taken is to incorporate the spores and crystals of B.t. PS50C into bait granules containing an attractant and applying these granules to the soil for control of soil-inhabiting Coleoptera. Formulated B.t. PS50C can also be applied as a seed-coating or root treatment or total plant treatment.

55

The B.t. PS50C cells can be treated prior to formulation to prolong the pesticidal activity when the cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not seriously affect the properties of the pesticide, nor diminish the cellular capability in protecting the pesticide. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, Iodine can be

used under mild conditions and for sufficient time to achieve the desired results. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Bouin's fixative and Helly's fixative (See: Humason, Gretchen L., *Animal Tissue Techniques*, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of the target pest(s). Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like.

The novel toxin gene of the subject invention was obtained from a novel coleopteran-active *B. thuringiensis* (B.t.) isolate designated B.t. PS50C. The gene was isolated as disclosed in the Examples.

The toxin gene of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., *Pseudomonas*, the microbes can be applied to the situs of coleopteran insects where they will proliferate and be ingested by the insects. The result is a control of the unwanted insects. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of target pest(s). The resulting product retains the toxicity of the B.t. toxin.

Where the B.t. toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytopshere" (phyloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilus*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leucosporidium*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*. Of particular interest are such phytopshere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*, *Agrobacterium tumefaciens*, *Rhodopseudomonas sphaeroides*, *Xanthomonas campestris*, *Rhizobium meliloti*, *Alcaligenes entrophus*, and *Acetobacter vinelandii*; and phytopshere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantia*, *Cryptococcus albidus*, *C. diaffluens*, *C. laurentii*, *Saccharomyces roseus*, *S. prattiensis*, *S. cerevisiae*, *Sporobolomyces roseus*, *S. odonis*, *Kluyveromyces veronae*, and *Aureobasidium pullulans*. Of particular interest are the pigmented microorganisms.

A wide variety of ways are available for introducing the B.t. gene expressing the toxin into the microorganism host under conditions which allow for stable maintenance and expression of the gene. One can provide for DNA constructs which include the transcriptional and translational regulatory signals for expression of the toxin gene, the toxin gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

The transcriptional initiation signals will include a promoter and a transcriptional initiation start site. In some instances, it may be desirable to provide for regulative expression of the toxin, where expression of the toxin will only occur after release into the environment. This can be achieved with operators or a region binding to an activator or enhancers, which are capable of induction upon a change in the physical or chemical environment of the microorganisms. For example, a temperature sensitive regulatory region may be employed, where the organisms may be grown up in the laboratory without expression of a toxin, but upon release into the environment, expression would begin. Other techniques may employ a specific nutrient medium in the laboratory, which inhibit the expression of the toxin, where the nutrient medium in the environment would allow for expression of the toxin. For translational initiation, an ribosomal binding site and an initiation codon will be present.

Various manipulations may be employed for enhancing the expression of the messenger, particularly by using an active promoter, as well as by employing sequences, which enhance the stability of the messenger RNA. The initiation and translational termination region will involve stop codon(s), a terminator region, and optionally, a polyA' polyadenylation signal.

In the direction of transcription, namely in the 5' to 3' direction of the coding or sense sequence, the construct will involve the transcriptional regulatory region, if any, and the promoter, where the regulatory region

may be either 5' or 3' of the promoter, the ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker, where the second DNA sequence may be joined to the toxin expression construct during introduction of the DNA into the host.

By a marker is intended a structural gene which provides for selection of those hosts which have been modified or transformed. The marker will normally provide for selective advantage, for example, providing for blood resistance, e.g., resistance to antibiotics or heavy metals; complementation, so as to provide prototrophy to an auxotrophic host, or the like. Preferably, complementation is employed, so that the modified host may not only be selected, but may also be competitive in the field. One or more markers may be employed in the development of the constructs, as well as for modifying the host. The organisms may be further modified by providing for a competitive advantage against other wild-type microorganisms in the field. For example, genes expressing metal chelating agents, e.g., siderophores, may be introduced into the host along with the structural gene expressing the toxin. In this manner, the enhanced expression of a siderophore may provide for a competitive advantage for the toxin-producing host, so that it may effectively compete with the wild-type microorganisms and stably occupy a niche in the environment.

Where no functional replication system is present, the construct will also include a sequence of at least 50 basepairs (bp), preferably at least about 100 bp, and usually not more than about 1000 bp of a sequence homologous with a sequence in the host. In this way, the probability of legitimate recombination is enhanced, so that the gene will be integrated into the host and stably maintained by the host. Desirably, the toxin gene will be in close proximity to the gene providing for complementation as well as the gene providing for the competitive advantage. Therefore, in the event that a toxin gene is lost, the resulting organism will be likely to also lose the complementing gene and/or the gene providing for the competitive advantage, so that it will be unable to compete in the environment with the gene retaining the intact construct.

A large number of transcriptional regulatory regions are available from a wide variety of microorganisms, hosts, such as bacteria, bacteriophage, cyanobacteria, algae, fungi, and the like. Various transcriptional regulatory regions include the regions associated with the *tsp* gene, *lac* gene, *gal* gene, the lambda left and right promoters, the *cba* promoter, the naturally-occurring promoters associated with the toxin gene, where functional in the host. See for example, U.S. Patent Nos. 4,332,898, 4,342,832 and 4,356,270. The termination region may be the termination region normally associated with the transcriptional initiation region or a different transcriptional initiation region, so long as the two regions are compatible and functional in the host.

Where stable episomal maintenance or integration is desired, a plasmid will be employed which has a replication system which is functional in the host. The replication system may be derived from the chromosome, an episomal element normally present in the host or a different host, a replication system from a virus which is stable in the host. A large number of plasmids are available, such as pBR322, pACYC184, RSF1010, pRK1014, and the like. See for example, Olson et al., (1982) J. Bacteriol. 150:8069, and Bagdasarian et al., (1981) Gene 16:237, and U.S. Patent Nos. 4,356,270, 4,362,817, and 4,371,825.

The **B1** gene can be introduced between the transcriptional and translational initiation region and the transcriptional and translational termination region, so as to be under the regulatory control of the initiation region. This construct will be included in a plasmid, which will include at least one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, which have been described previously. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for pesticidal activity.

Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin in the cell when then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and positive, include Enterobacteriaceae, such as *Escherichia*, *Erwinia*, *Shigella*, *Salmonella*, and *Proteus*; *Bacillaceae*; *Rhizobiaceae*, such as *Rhizobium*; *Spizellaceae*, such as *photobacterium*, *Zymomonas*, *Serratia*, *Aerom us*, *Nasuta*, *Vibrio*, *Desulfobulvri*, *Spirillum*; *Lactobacillaceae*; *Pseudomonadaceae*, such as *Pseudomonas* and *Acetobacter*; *Azotobacteraceae* and

Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the B.t. gene into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, stability, and the like.

Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phytoplano organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms as Escherichia, Lactobacillus sp., Bacillus sp., Streptomyces sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, Streptomyces lividans, and the like.

The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

Treatment of the recombinant microbial cell can be done as disclosed infra. The treated cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of inactivation should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

The cellular host containing the B.t. insecticidal gene may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the B.t. gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

The B.t. cells may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllsilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

The pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-80% by weight of the solids in the liquid phase. The formulations will generally have from about 10<sup>3</sup> to about 10<sup>4</sup> cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

The formulations can be applied to the environment of the coleopteran pest(s), e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

#### Example 1 - Culturing B.t. PS50C, NRRL B-18746

A subculture of B.t. PS50C, NRRL B-18746 can be used to inoculate the following medium, a peptone, glucose, salts medium.

Bacto Peptone	7.5 g/l
Glucose	1.0 g/l
KH <sub>2</sub> PO <sub>4</sub>	3.4 g/l
K <sub>2</sub> HPO <sub>4</sub>	4.35 g/l
Salt Solution	5.0 ml/l
CaCl <sub>2</sub> Solution	5.0 ml/l
Salts Solution (100 ml)	

MgSO<sub>4</sub>.7H<sub>2</sub>O 2.46 g  
 MnSO<sub>4</sub>.H<sub>2</sub>O 0.04 g  
 ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.28 g  
 FeSO<sub>4</sub>.7H<sub>2</sub>O 0.40 g  
 5 CaCl<sub>2</sub> Solution (100 ml)  
 CaCl<sub>2</sub>.2H<sub>2</sub>O 3.66 g  
 pH 7.2

The salts solution and CaCl<sub>2</sub> solution are filter-sterilized and added to the autoclaved and cooled broth at the time of inoculation. Flasks are incubated at 30°C on a rotary shaker at 200 rpm for 64 hr.

10 The above procedure can be readily scaled up to large fermentors by procedures well known in the art.  
 The *B.t.* spores and crystals, obtained in the above fermentation, can be isolated by procedures well known in the art. A frequently-used procedure is to subject the harvested fermentation broth to separation techniques, e.g., centrifugation.

15 Example 2 - Testing of *B.t.* PS50C, NRRL B-18746 Spores and Crystals

B.t. PS50C, NRRL B-18746 spores and crystals are toxic to the Colorado potato beetle (CPB). The assay for the Colorado potato beetle was conducted as follows:

CPB Bioassay - Early second instar larvae of Leptinotarsa decemlineata are placed on potato leaves which have been dipped in suspensions containing *Bacillus thuringiensis* preparations. The larvae are incubated at 25°C for 4 days, and larval mortality is recorded and analyzed using probit analysis.

Example 3 - Cloning of a Novel Toxin Gene from *B.t.* Isolate PS50C

25 Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells grown to an optical density, at 600 nm, of 1.0. The cells were recovered by centrifugation and protoplasts were prepared in TES buffer (30 mM Tris-HCl, 10 mM EDTA 50 mM NaCl, pH = 8.0) containing 20% sucrose and 50 mg/ml lysozyme. The protoplasts were lysed by addition of SDS to a final concentration of 4%. The cellular material was precipitated overnight at 4°C in 100 mM (final concentration) neutral potassium chloride. The supernate was extracted twice with phenol/chloroform (1:1). Nucleic acids were precipitated with ethanol and DNA was purified by isopycnic banding on cesium chloride-ethidium bromide gradients.

Total cellular DNA from *B.t.* subsp. kumamotoensis (*B.t.kum.*), isolate PS50C, was digested with HindIII and fractionated by electrophoresis on a 0.8% (w/v) agarose-TAE (50 mM Tris-HCl, 20 mM NaOAc, 2.5 mM EDTA, pH = 8.0) buffered gel. A Southern blot of the gel was hybridized with a [<sup>32</sup>P]-radiolabeled oligonucleotide probe. Results showed that the hybridizing fragments of PS50C are approximately 12 Kb and 1.7 Kb in size.

35 A library was constructed from PS50C total cellular DNA partially digested with Sau3A and size fractionated by gel electrophoresis. The 9-23 Kb region of the gel was excised and the DNA was electroeluted and then concentrated using an Elutip-d™ ion exchange column (Schleicher and Schuel, Keene, NH). The isolated Sau3A fragments were ligated into BamH-digested LambdaGEM-11™ (PROMEGA). The packaged phage 40 probe was plated on *E. coli* KW251 cells (PROMEGA) at a high titer and screened using the radiolabelled oligonucleotide probe. Hybridizing plaques were purified and rescreened at a lower plaque density. Single isolated, purified plaques that hybridized with the probe were used to infect *E. coli* KW251 cells in liquid culture for preparation of phage for DNA isolation. DNA was isolated by standard procedures. Preparative amounts of DNA were digested with XbaI (to release the inserted DNA from lambda sequences) and separated by 45 electrophoresis on a 0.6% agarose-TAE gel. The large fragments were purified by ion exchange chromatography as above and ligated to XbaI-digested, dephosphorylated pHTBlueII (an *E. coli*/*B. thuringiensis* shuttle vector comprised of pBluescript skF [Stratagene] and the replication origin from a resident *B.t.* plasmid [D. Lereclus et al. 1989, FEMS Microbiology Letters 60:211-218]). The ligation mix was introduced by transformation into competent *E. coli* NM522 cells (ATCC 47000) and plated on LB agar containing ampicillin, isopropyl-( $\beta$ -D-thiogalactoside (IPTG) and 5-bromo-4-chloro-4-indolyl-( $\beta$ -D-galactoside (XGAL). White colonies, with putative restriction fragment insertions in the ( $\beta$ -D-galactosidase gene of pHTBlueII), were subjected to standard rapid plasmid purification procedures. Plasmids were analyzed by XbaI digestion and agarose gel electrophoresis. The desired plasmid construct, pMYC1638, contains an approximately 12 Kb XbaI insert. A partial restriction map (see drawing) of the cloned insert indicates that the toxin gene is novel compared to the 50 maps of other toxin genes encoding insecticidal proteins. The nucleotide sequence (open reading frame only) is shown in Sequence ID No. 1. The predicted peptide sequence of the toxin is shown in Sequence ID No. 2. Plasmid pMYC1638 was introduced into an acrystalliferous (*Cry*) *B.t.* host (HD-1 *cry*B obtained from A. Aronson, Purdue University) by electroporation. Expression of an approximately 130 kDa protein was verified

by SDS-PAGE. Broth containing spores and crystals was used for the determination of toxicity to Lepidotarsa decemlineata.

5 Plasmid pMYC1638 containing the B.t toxin gene, can be removed from the transformed host microbe by use of standard well-known procedures. For example, E. coli NM522[pMYC1638] NRRL B-18751 can be subjected to cleared lysate isopycnic density gradient procedures, and the like, to recover pMYC1638.

Example 4 - Insertion of Toxin Gene into Plants

10 The novel gene coding for the novel insecticidal toxin, as disclosed herein, can be inserted into plant cells using the Ti plasmid from Agrobacter tumefaciens. Plant cells can then be caused to regenerate into plants (Zambryski, P., Joos, H., Gentello, C., Leemans, J., Van Montagu, M. and Schell, J [1983] Cell 32:1033-1043). A particularly useful vector in this regard is pEND4K (Kee, H.J., Yanofsky, M.F. and Nester, E.W. [1985] BioTechnology 3:637-642). This plasmid can replicate both in plant cells and in bacteria and has multiple cloning sites for passenger genes. The toxic gene, for example, can be inserted into the BamH I site of pEND4K propagated in E. coli, and transformed into appropriate plant cells.

Example 5 - Cloning of Novel B. thuringiensis Gene Into Baculoviruses

20 The novel gene of the invention can be cloned into baculoviruses such as Autographa californica nuclear polyhedrosis virus (AcNPV). Plasmids can be constructed that contain the AcNPV genome cloned into a commercial cloning vector such as pUC8. The AcNPV genome is modified so that the coding region of the polyhedrin gene is removed and a unique cloning site for a passenger gene is placed directly behind the polyhedrin promoter. Examples of such vectors are pGP-86874, described by Pennock et al. (Pennock, G.D., Shoemaker, C. and Miller, L.K. [1984] Mol. Cell. Biol. 4:399-406), and pAC380, described by Smith et al. (Smith, G.E., Summers, M.D. and Fraser, M.J. [1983] Mol. Cell. Biol. 3:2158-2165). The gene coding for the novel protein toxin of the invention can be modified with BamH I linkers at appropriate regions both upstream and downstream from the coding region and inserted into the passenger site of one of the AcNPV vectors.

30

35

40

45

50

## SEQUENCE LISTING

5

10

15

20

25

(1) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3471 base pairs
  - (B) NUCLEOTIDESEGMENT: 1
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Bacillus thuringiensis*
  - (B) STRAIN: kumamotoensis
  - (C) INDIVIDUAL ISOLATE: P50C
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: LAMSDAGEM (TM) - 11 LIBRARY OF LUIS FONCERADA
  - (B) CLONE#: 50C
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAGTCCAA ATATACAAA TGATATGAA ATTATAGATG CGACACCTTC TACATCTGTA	60
TCCACTGATT CTAAACAGATA CCCTTTGCG ATTGAGGCCAA CAGATGCGTT ACAAAATATG	120
AATTATATAG ATTATCTGAA ATATGCTGGG GGAGAGAACTC CTGAATTATATT TGGAAATCCG	180
GAGACGTTTA TTAGTTCATC CACGATTCAA ACTGGGATTG GCATTGTTGG TCGAAATACTA	240
GGAGCTTTAG GGGTTCCATT TGCCTAGTCAG ATAGCTGTTT TCCTATAGTTT CATTGTTGGT	300
CAATTATGGC CGTCAGATATA TGGGGAAA TTATGGAACG AGTGGAGAG	360

40

50

55

CTCTGGATC AAAAATAGA AATAATGTA AAAGATRAGG CTCTTGCTGA ATTAAAAGGG 420  
 CTAGGAATG CTTGGATCT ATATCAGCG TCACITGAA AGTGGCTGGA AATTCGCAT 480  
 5 GATGCAAGAA CTAGAAGTGT GTTTCCTAAT CAATTATAGA CTTTAGATCT TAACTTTGTT 540  
 AGTCAATTC CATCTTTGC AGTATCCGGA CACGAAGTAC TATTATTTAGC AGTATATGCA 600  
 CAGGGCTGGA ACCTACATTT ATTCGTTATA AGACATGCTT CTATTTTTGG AGACAGTGG 660  
 GGATTTAACAC CAGGTGAAT TCTAGATTT AATACTGCA AGTGCACAT TACCGCTGAA 720  
 10 TATTCAGACT ATTCGTTAAA GTGGTATAAA ATCCGGCTTAG ATAATATGAA AGTACCACT 780  
 TCTAAAAGTT GGCTGAATTA TCATCAAGTTC CGTAGAGAGA TGACATTACT GGTTAGAT 840  
 TTGGTGCGGT TATTTCCAAA GTATGACACA CATAATGATC CAATCGAAC AACAGCTCAA 900  
 15 CTTCACCGG AGTGTATACAG AGATCCGATA CCAATTCATA TAGTGACANG TACTGGATTC 960  
 TGCACACCCCTT GGTCACACCCAGTGGTATCTT CTTTATGATC AGTTGAAAAA CAAACGTAATT 1020  
 CGTCGGCCAC ATCTTGTGA TATACATCAGC TCAGTGAAGAA TTAAATCACAG TAGGGGGGGT 1080  
 ATTACGTTAA ATATGTATGC ATATATAAAC TACTGGTCAG GACATACCCCT AAAATATGTT 1140  
 AGAACAGCTT ATTCGACCGT AACATACACA GCTAACTTACG GTGCAATTCAC TTCAGAAG 1200  
 20 AATTCAATTG CACTTGAGGA TGGGGATTTT TTGGAATTTA ATTCACACTG GGCAAAACCTA 1260  
 GCTAATTACT ACCAAAAGGG ATATGGTGTG CGGGGATCTT GTGTCATATG GTGAAANAGG 1320  
 GGAACCTCAT AACACACAGC GTATTTATAT TCAAAACAC ATACAGCTCT CCAAGGGTGT 1380  
 ACACAGGTTT ATGATACAG TGATGAAATA CCTCTATGATA GAATGTTACCC GGTTAGCTGAA 1440  
 25 AGCTTATGTC ATAGATTTAC TCATATTCTC TCCCATCTT TCTCTAAAAA TGGGACTGCA 1500  
 TACTTATGGGA GTTTCCTGTG ATTTGTTGG ACACATACATA GTGGGGATT AAAATAATACA 1560  
 ATATATTCTG ATAAAATCAC TCAAAATCCA CGGGTAAAGG GAGACATGTT ATATCTAGGG 1620  
 GTGTCGGTATC TACAGGGTCC TGGGATACAGA GGAGGAGATA TATTAAGAAAG ACCAAATCTT 1680  
 30 AGCATATTAG GGACCTTTCG GGTGATGATA ATGGGGTGT TATCACAAG ATATGTTGTA 1740  
 AGAAATTGGCT ATGCCCTCAC AACAGATTTT GAAATTACTC TATACCTTGG CGACACAAATA 1800  
 GAAAATATAA GTNTTAAACA AACTATGGAT ATGGGGCAT CTTTAACGTA TGAAACATT 1860  
 AATTCGCGAA GTTTCGTCG TGATTTCCAA TTCAGAGATA AACAGATAAA ATACTCCCTA 1920  
 35 TCCATGGGTG ATTTAGTC CCGTCAGAAA GTTATATTAAG ACCGAATCGA ATTCATCCCCA 1980  
 GTAGATGAGA CATATGAGG CGAACACAGAT TTAGAGACGG CGAAGAAAGC AGTGAATGCC 2040  
 TTGTTTACGA ATACAAAAGA TGGCTTAAGA CCAGGTGTTA CGGATTATGAA AGTAAATCAA 2100  
 GCGGCAACT TACTGGGGT CCTATCGGT GTTATATACG CAAATGAAA ACCATTGTTA 2160  
 40 TTGATGCGG TGAGAGGGC AAAACGCCCTC ACTGGGGCAC GTAACTTACT AACAGATCCA 2220  
 GATTCCCGAG AGATAAACGG AGAAANATGGA TGGGGCGCAA GTACGGGAAT TGAGATGTTA 2280  
 GAAGGGGGATG CTGTATTTAA AGCGAGTTAT CTACGCCATC CAGGTGCAAG AGAAATTTGAT 2340  
 ACGGAACAGT ATCCAACTG TCTGTATCAA AAAGTCAAGG AGGTGTATT AAAACCATAC 2400  
 45 ACAAGATATA GACTGAGGG TTGTTGGGA AGTACTGCAAG GATTGAAAT TTATACGATA 2460  
 CGTCACCCAA CGAAATGGAT TGTAAAGGAT GTACCGAGAT ATTTATGCCC AGATGTTCT 2520  
 CCTGTAAACT CTGATGGCAG TATCAGTCGA TGCAGCGAAC AAAAGTATGTT GAATAGCCGT 2580  
 TTGAAAGGAG AARACCGGTC TGGTGTATGCA CAGTGGTTCT CGCTCCCTAT CGATATAGGA 2640  
 50 GAGCTGGATT AACATGAAATA TCCAGGATAA TGGGGTGGAA TTAAGATTAC CGACCCACAG 2700

GGATACGGAA CACTTGGAAA TCTTGAAITA GTGGAGAGG GACCCTTGTG AGGAGAGCA 2760  
 5 TTAGACCGGT TGCRAAGGGA AGAACACAG TGGAAGAGTC AAATGACAAAG AAGACGTGAA 2820  
 GAGACAGATA GAAGATACAT GGCACTCCAAA CAACCGGTAG ATCGTTATA TCCCATTAT 2880  
 CAGGATCACG AACTGAATCC TGATGTAGAG ATTACAGATC TTACTGGGC TCAAGATCTG 2940  
 ATACAGTCAA TTCCCTTACGT ATATAACGAA ATGTTCCCAAA AAATACCGGG GATGAACAT 3000  
 ACCGAGTTTA CAGAATTAAAC AGATGAGCTC CAACRAGCGT GGAATTGTA TGATCAGCGA 3060  
 10 ATGCCCCATAC CAAATGGTGA TTTCGAAAT GGGTTTAACTG ATGGAAATGG AACGCCCTGGC 3120  
 GTAGAAAGTAC ARCAAATCAA TCATACATCT GTCCCTGGTA TTCCAAACTG GGATGAAACAA 3180  
 GTTTCACAC AGTTTACAGT TCAACCGAAAT CAAAGATATG TATTACAGGT TACTGCAAGA 3240  
 AAAGAAGGGG TAGGAAATGG ATATGTAGT ATTGGTGTAG GGGAAATCA ATCAGAAAACG 3300  
 15 CTTACTTTTA GTGCAAGCGA TTATGATACA AATGGTGTGT ATAATGACCA AACCCGCTAT 3360  
 ATCACAAAAA CAGTGACATC CATCCCGTAT ACAGATCAAAT TGTTGATGAA AATTAAGTGA 3420  
 ACAGAAGGTA COTTCTATAT AGAAGATGTA GAATTGATG TAGACGTAGA G 3471

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1157 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

- (iii) HYPOTHETICAL: YES

- (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus thuringiensis*
- (B) ISOLATION: KUNMING, CHINA
- (C) INDIVIDUAL ISOLATE: PS50C

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Lambdagem (TM) - 11 LIBRARY OF LUIS FONCERRADA

- (B) CLONE: 50C

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Pro	Asn	Asn	Gln	Asn	Glu	Tyr	Ile	Ile	Asp	Ala	Thr	Pro	
1									5	10				15	
Ser	Thr	Ser	Val	Ser	Ser	Asp	Ser	Asn	Arg	Tyr	Pro	Phe	Ala	Asn	Glu
	20							25					30		
Pro	Thr	Asp	Ala	Leu	Gln	Asn	Met	Asn	Tyr	Lys	Asp	Tyr	Leu	Lys	Met
	35						40				45				
Ser	Gly	Gly	Glu	Asn	Pro	Glu	Leu	Phe	Gly	Asn	Pro	Glu	Thr	Phe	Ile
	50						55			60					
Ser	Ser	Ser	Thr	Ile	Cln	Thr	Gly	Ile	Gly	Ile	Val	Gly	Arg	Ile	Ile
	65						70			75				80	
Gly	Ala	Leu	Gly	Val	Pro	Phe	Ala	Ser	Gln	Ile	Ala	Ser	Phe	Tyr	Ser
	85						90			95					
Phe	Ile	Val	Gly	Gln	Leu	Trp	Pro	Ser	Lys	Ser	Val	Asp	Ile	Trp	Gly
	100						105			110					
Glu	Ile	Met	Glu	Arg	Val	Glu	Glu	Leu	Val	Asp	Gln	Lys	Ile	Glu	Lys
	115						120			125					

50

55

Tyr Val Lys Asp Lys Ala Leu Ala Glu Leu Lys Gly Leu Gly Asn Ala  
 130 135 140  
 Leu Asp Val Tyr Ile Gln Ser Leu Glu Asp Trp Leu Glu Asn Arg Asn  
 145 150 155 160  
 Asp Ala Arg Thr Arg Ser Val Val Ser Asn Gln Phe Ile Ala Leu Asp  
 165 170 175  
 Leu Asn Phe Val Ser Ser Ile Pro Ser Phe Ala Val Ser Gly His Glu  
 180 185 190  
 Val Leu Leu Ala Val Tyr Ala Gln Ala Val Asn Leu His Leu Leu  
 195 200 205  
 Leu Leu Arg Asp Ala Ser Ile Phe Gly Glu Glu Trp Gly Phe Thr Pro  
 210 215 220 225  
 Gly Glu Ile Ser Arg Phe Tyr Asn Arg Gln Val Gln Leu Thr Ala Glu  
 225 230 235 240  
 Tyr Ser Asp Tyr Cys Val Lys Trp Tyr Lys Ile Gly Leu Asp Lys Leu  
 245 250 255  
 Lys Gly Thr Thr Ser Lys Ser Trp Leu Asn Tyr His Gln Phe Arg Arg  
 260 265 270  
 Glu Met Thr Leu Leu Val Leu Asp Leu Val Ala Leu Phe Pro Asn Tyr  
 275 280 285  
 Asp Thr His Met Tyr Pro Ile Glu Thr Thr Ala Gln Leu Thr Arg Asp  
 290 295 300  
 Val Tyr Thr Asp Pro Ile Ala Phe Asn Ile Val Thr Ser Thr Gly Phe  
 305 310 315 320  
 Cys Asn Pro Trp Ser Thr His Ser Gly Ile Leu Phe Tyr Glu Val Glu  
 325 330 335  
 Asn Asn Val Ile Arg Pro Pro His Leu Phe Asp Ile Leu Ser Ser Val  
 340 345 350  
 Glu Ile Asn Thr Ser Arg Gly Gly Ile Thr Leu Asn Asn Asp Ala Tyr  
 355 360 365  
 Ile Asn Tyr Trp Ser Gly His Thr Leu Lys Tyr Arg Arg Thr Ala Asp  
 370 375 380  
 Ser Thr Val Thr Tyr Thr Ala Asn Tyr Gly Arg Ile Thr Ser Glu Lys  
 385 390 395 400  
 Asn Ser Phe Ala Leu Glu Asp Arg Asp Ile Phe Glu Ile Asn Ser Thr  
 405 410 415  
 Val Ala Asn Leu-Ala Asn Tyr Tyr Glu Lys Ala Tyr Gly Val Pro Gly  
 420 425 430  
 Ser Trp Phe His Met Val Lys Arg Gly Thr Ser Ser Thr Thr Ala Tyr  
 435 440 445  
 Leu Tyr Ser Lys Thr His Thr Ala Leu Gln Gly Cys Thr Gln Val Tyr  
 450 455 460 465  
 Glu Ser Ser Asp Glu Ile Pro Leu Asp Arg Thr Val Pro Val Ala Glu  
 470 475 480  
 Ser Tyr Ser His Arg Leu Ser His Ile Thr Ser His Ser Phe Ser Lys  
 485 490 495  
 Asn Gly Ser Ala Tyr Tyr Gly Ser Phe Pro Val Phe Val Thr His  
 500 505 510  
 Thr Ser Ala Asp Leu Asn Asn Thr Ile Tyr Ser Asp Lys Ile Thr Gln  
 515 520 525  
 Ile Pro Ala Val Lys Gly Asp Met Leu Tyr Leu Gly Gly Ser Val Val  
 530 535 540

545 Gln Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Lys Arg Thr Asn Pro  
 550 555 560  
 Ser Ile Leu Gly Thr Phe Ala Val Thr Val Asn Gly Ser Leu Ser Gln  
 565 570 575  
 5 Arg Tyr Arg Val Arg Ile Arg Tyr Ala Ser Thr Thr Asp Phe Glu Phe  
 580 585 590  
 Thr Leu Tyr Leu Gly Asp Thr Ile Glu Lys Asn Arg Phe Asn Lys Thr  
 595 600 605  
 Met Asp Asn Gly Ala Ser Leu Thr Tyr Glu Thr Phe Lys Phe Ala Ser  
 610 615 620  
 Phe Ile Thr Asp Phe Gln Phe Arg Glu Thr Gln Asp Lys Ile Leu Leu  
 625 630 635 640  
 Ser Met Gly Asp Phe Ser Ser Gly Gln Glu Val Tyr Ile Asp Arg Ile  
 645 650 655  
 16 Glu Phe Ile Pro Val Asp Glu Thr Tyr Glu Ala Glu Gln Asp Leu Glu  
 660 665 670  
 Ala Ala Lys Ala Val Asn Ala Leu Phe Thr Asn Thr Lys Asp Gly  
 675 680 685  
 Leu Arg Pro Gly Val Thr Asp Tyr Glu Val Asn Gln Ala Ala Asn Leu  
 690 695 700  
 20 Val Glu Cys Leu Ser Asp Asp Leu Tyr Pro Asn Glu Lys Arg Leu Leu  
 705 710 715 720  
 Phe Asp Ala Val Arg Glu Ala Lys Arg Leu Ser Gly Ala Arg Asn Leu  
 725 730 735  
 25 Leu Gln Asp Pro Phe Gln Glu Ile Asn Gly Glu Asn Gly Trp Ala  
 740 745 750  
 Ala Ser Thr Gly Ile Glu Ile Val Glu Gly Asp Ala Val Phe Lys Gly  
 755 760 765  
 Arg Tyr Leu Arg Leu Pro Gly Ala Arg Glu Ile Asp Thr Glu Thr Tyr  
 770 775 780  
 30 Pro Thr Tyr Leu Tyr Gln Lys Val Glu Glu Gly Val Leu Lys Pro Tyr  
 785 790 795 800  
 Thr Arg Tyr Arg Leu Arg Gly Phe Val Gly Ser Ser Gln Gly Leu Glu  
 805 810 815  
 35 Ile Tyr Thr Ile Arg His Cln Thr Asn Arg Ile Val Lys Asn Val Pro  
 820 825 830  
 Asp Asp Leu Leu Pro Asp Val Ser Pro Val Asn Ser Asp Gly Ser Ile  
 835 840 845  
 Asn Arg Cys Ser Glu Gln Lys Tyr Val Asn Ser Arg Leu Glu Gly Glu  
 850 855 860  
 40 Asn Arg Ser Gly Asp Ala His Glu Phe Ser Pro Ile Asp Ile Gly  
 865 870 875 880  
 Glu Leu Asp Tyr Asn Glu Asn Ala Gly Ile Trp Val Gly Phe Lys Ile  
 885 890 895  
 Thr Asp Pro Glu Gly Tyr Ala Thr Leu Gly Asn Leu Glu Leu Val Glu  
 900 905 910  
 45 Glu Gly Pro Leu Ser Gly Asp Ala Leu Glu Arg Leu Gln Arg Glu Glu  
 915 920 925  
 Gln Gln Trp Lys Ile Gln Met Thr Arg Arg Arg Glu Glu Thr Asp Arg  
 930 935 940  
 50 Arg Tyr Met Ala Ser Iys Gln Ala Val Asp Arg Leu Tyr Ala Asp Tyr  
 945 950 955 960

Gln Asp Gln Gln Leu Asn Pro Asp Val Glu Ile Thr Asp Leu Thr Ala  
 965 970 975  
 Ala Gln Asp Lu Ile Gln Ser Ile Pro Tyr Val Tyr Asn Lu Met Phe  
 980 985 990  
 5 Pro Glu Ile Pro Gly Met Asn Tyr Thr Lys Phe Thr Glu Leu Thr Asp  
 995 1000 1005  
 Arg Leu Glu Gln Ala Trp Asn Leu Tyr Asp Gln Arg Asn Ala Ile Pro  
 1010 1015 1020  
 10 Asn Gly Asp Phe Arg Asn Gly Leu Ser Asn Trp Asn Ala Thr Pro Gly  
 1025 1030 1035 1040  
 15 Val Glu Val Gln Gln Ile Asn His Thr Ser Val Leu Val Ile Pro Asn  
 1045 1050 1055 1060  
 Trp Asp Glu Gln Val Ser Gln Phe Thr Val Glu Pro Asn Gln Arg  
 1060 1065 1070  
 Tyr Val Leu Arg Val Thr Ala Arg Lys Glu Gly Val Gly Asn Gly Tyr  
 1075 1080 1085  
 Val Ser Ile Arg Asp Gly Gly Asn Glu Ser Glu Thr Leu Thr Phe Ser  
 1090 1095 1100  
 20 Ala Ser Asp Tyr Asp Thr Asn Gly Val Tyr Asn Asp Glu Thr Gly Tyr  
 1105 1110 1115 1120  
 Ile Thr Lys Thr Val Thr Phe Ile Pro Tyr Thr Asp Glu Met Trp Ile  
 1125 1130 1135  
 Glu Ile Ser Glu Thr Glu Gly Thr Phe Tyr Ile Glu Ser Val Glu Leu  
 1140 1145 1150  
 25 Ile Val Asp Val Glu  
 1155

## Claims

- 30 1. A culture of a microorganism which is Bacillus thuringiensis PS50C, having the identifying characteristics of NRRL B-18746, or a mutant thereof, having activity against insect pests of the order Coleoptera.
2. A composition comprising a microorganism as defined in claim 1, or spores or crystals thereof, in association with an insecticide carrier.
3. A composition according to claim 2, which comprises beetle phagostimulants or attractants.
4. A composition according to claim 2 or claim 3, which comprises formulation ingredients applied as a seed coating.
- 40 5. A composition according to any of claims 2 to 4, in the form of a bait granule.
6. A toxin, active against coleopteran pests, having a molecular weight of c. 130 kDa and the amino-acid sequence shown in Sequence ID No. 2.
- 45 7. DNA encoding a toxin according to claim 6.
8. DNA having the nucleotide sequence shown in Sequence ID No. 1.
9. A recombinant DNA transfer vector comprising DNA according to claim 7 or claim 8.
- 50 10. A host transformed with a DNA transfer vector according to claim 9.
11. A bacterial host transformed to express a toxin according to claim 6.
- 65 12. A host according to claim 10 or claim 11, which is Escherichia coli.
13. A host according to claim 12, which is Escherichia coli NM522 (pMYC1638), as available from NRRL, Accession No. B-18751.

14. A host according to claim 11, which is a species of Pseudomonas, Azotobacter, Erwinia, Serratia, Klebsiella, Rhizobium, Bacillus, Streptomyces, Rhodopseudomonas, Methylphilus, Agrobacterium, Acetobacter or Alcaligenes.

5 15. A host according to claim 14, which is pigmented and phylloplane-adherent.

16 16. A host according to claim 11, which is a root-colonising bacterium.

17. Substantially intact, treated cells, having prolonged pesticidal activity when applied to the environment of  
10 a target pest, containing, intracellularly, a toxin according to claim 6.

18. Cells according to claim 17, which are prokaryotes or lower eukaryotes.

19. Cells according to claim 18, which are prokaryotic cells selected from Enterobacteriaceae, Bacillaceae,  
15 Rhizobiaceae, Spriilaceae, Lactobacillaceae, Pseudomonadaceae, Azotobacteraceae and Nitrobac-  
teraceae, or eukaryotic cells selected from Phycomycetes, Ascomycetes and Basidiomycetes.

20. Cells according to claim 17, which are of a pigmented bacterium, yeast or fungus.

21. Cells according to claim 17, of a unicellular microorganism.

22. Cells according to claim 20, wherein the microorganism is Pseudomonas.

23. Cells according to claim 22, of Pseudomonas fluorescens.

24. Cells according to any of claims 17 to 23, which are treated with Iodine.

25. The plasmid denoted pMYC1638, as available from NRRL, Accession No. B-18751.

26. A process for controlling a coleopteran insect pest, which comprises administering to the pest or the envi-  
ronment thereof, a microorganism as defined in claim 1, a composition according to any of claims 2 to 5,  
30 a toxin according to claim 6, a host according to any of claims 10 to 16, or cells according to any of claims  
17 to 24.

27. A process according to claim 26, wherein the pest is soil-inhabiting, which comprises applying a bait  
granule according to claim 5 to or into the soil.

35 28. A process according to claim 27, wherein the bait granule is applied at the same time corn seed is planted  
in the soil.

29. A process according to claim 28, wherein the pests are present on stored products.

40 30. A process according to claim 26, wherein the administration is to the rhizosphere, the phylloplane or a  
body of water.

31. A process according to any of claims 26 to 30, wherein the pest is the Colorado potato beetle.

45

50

55

